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STIMULATION OF $^{22}Na^+$ EFFLUX FROM RAT FOREBRAIN MEMBRANE VESICLES BY L-GLUTAMIC ACID, L-ASPARTIC ACID AND KAINIC ACID

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Abstract—A glass fiber filter assay method is described for measuring $^{22}Na^+$ efflux stimulated by L-glutamic acid, L-aspartic acid and kainic acid from osmotically sensitive membrane vesicles prepared from rat brain. L-Glutamic acid and L-aspartic acid showed the greatest efficacy for the stimulation of $^{22}Na^+$ efflux with EC_{50} values of 3 μM. Kainic acid produced 28% of the maximal efflux seen with L-glutamic acid or L-aspartic acid with an EC_{50} value of 1.5 μM. Quisqualic acid never showed statistically significant increases in $^{22}Na^+$ efflux over control experiments. *N*-Methyl-D-aspartic acid showed no detectable efflux activity in this preparation. DL-2-Amino-4-phosphonobutyric acid (APB) inhibited up to 40% of the 50 μM L-glutamic acid-stimulated or 50 μM L-aspartic acid-stimulated $^{22}Na^+$ efflux with an IC_{50} value of 1.5 nM. Calcium was required for the inhibitory action of APB, but not for the stimulatory actions of L-glutamic, L-aspartic, or kainic acids. L-Glutamic, L-aspartic, and kainic acids at concentrations above 100 μM were found to inhibit rather than to stimulate $^{22}Na^+$ efflux. Veratridine (1 μM) had no influence on the $^{22}Na^+$ efflux component which was produced by L-glutamic or kainic acids. We are unable to firmly establish the mechanism for the stimulated $^{22}Na^+$ efflux.

L-Glutamic acid and its agonists [the excitatory amino acids (EAAs)†] produce neuroexcitation throughout the mammalian CNS [1–3]. Much of the study of the neuroexcitatory actions of the EAAs has focused on glutamate receptor-mediated pharmacological and electrophysiological properties associated with alterations in transmembrane cation conductances [4, 5]. Glutamate receptor modulations of cyclic GMP formation [6, 7] and inositol phospholipid metabolism [8–11] have also been demonstrated. At least three major classes of CNS glutamate receptors have been differentiated based on their preferential activation by either quisqualic acid (QA), kainic acid (KA) or *N*-methyl-D-aspartic acid (NMDA) [1, 12, 13]. L-Glutamic acid and a number of other endogenous EAAs including aspartic acid, cysteic acid and cysteine sulfinic acid have been shown to be released from nerve terminals on depolarization, in a Ca^{2+}-dependent fashion [14–16]. L-Glutamic acid and L-aspartic acid are taken up from the extracellular space by both high and low affinity active uptake processes by both glial and neuronal cells [2, 17–20]. In glial cells a substantial proportion of the L-glutamic acid is converted to L-glutamine by the actions of glutamine synthetase [21–23]. L-Glutamine, released from glial cells, is readily taken up by synaptic terminals where it serves as a major metabolic precursor for the neurotransmitter pool of L-glutamic acid [24].

We were interested in examining the abilities of EAAs to influence the movement of Na^+ across CNS membranes. We found that micromolar concentrations of L-glutamic acid, L-aspartic acid and KA but not QA or NMDA were capable of stimulating the efflux (within 3 sec) of tracer quantities of $^{22}Na^+$ from rat brain membrane vesicle preparations *in vitro*. The EAA-stimulated $^{22}Na^+$ efflux was inhibited partially by nanomolar concentrations of DL-2-amino-4-phosphonobutyric acid (APB) which is reported to antagonize chloride-dependent glutamic acid uptake [17]. Glutamate receptor antagonists such as L-glutamic acid diethyl ester and DL-2-amino-5-phosphonovaleric acid (APV) were ineffective for inhibition of the EAA-stimulated Na^+ efflux. Veratridine, tetrodotoxin, and ouabain did not influence the EAA-stimulated component of the Na^+ efflux demonstrating that electrogenic sodium channels and Na^+,K^+-dependent ATPase activities were not directly involved in this activity. Amino acid–sodium cotransport or exchange mediated by EAA uptake and/or release processes alone or in combination with KA receptor-modulated ion channel events remain as viable mechanisms to explain this activity.

MATERIALS AND METHODS

L-Glutamic acid, L-aspartic acid, KA, QA, NMDA, APB, APV, L-glutamic acid diethyl ester, ouabain, glycine, glycylglycine, L-cysteine, L-cystine, 5-hydroxytyramine, γ-aminobutyric acid (GABA), veratridine, D-glucose, Ficoll, bovine serum albumin (BSA) and Trizma base were obtained from the Sigma Chemical Co., St. Louis, MO. Tetrodotoxin was obtained from the Sankyo Chemical Co. via Calbiochem-Behring, San Diego, CA. All other chemicals were of reagent quality. Long–Evans rats

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† Abbreviations: APB, DL-2-amino-4-phosphonobutyric acid; APV, DL-2-amino-5-phosphonovaleric acid; BSA, bovine serum albumin; EAA, excitatory amino acid; GABA, γ-aminobutyric acid; KA, kainic acid; NMDA, *N*-methyl-D-aspartic acid; and QA, quisqualic acid.

were purchased from Charles Rivers Breeding Laboratories, Inc., Wilmington, MA.

Membrane vesicle preparation. Forebrain from a freshly killed 200 g Long–Evans male rat was rinsed in ice-cold Na^+ buffer (130 mM NaCl, 5 mM Tris–HCl, 10 mM D-glucose, 1 mg/mL BSA, pH 7.4), transferred to 15 mL of fresh, ice-cold Na^+ buffer and homogenized with a 0.125 mm clearance glass–teflon homogenizer (6 up-and-down strokes at 300 rpm). The preparation was then fractionated by either density gradient ultracentrifugation or the whole-particulate fraction was collected by centrifugation.

Density gradient fractionation. The 15-mL homogenate was loaded onto the top of a discontinuous density gradient consisting of 8 mL of 16% (w/v) Ficoll 400,000 in choline buffer (130 mM choline chloride, 5 mM Tris–HCl, 10 mM D-glucose, 1 mg/mL BSA, pH 7.4), overlayered with 8 mL of 14% (w/v) Ficoll 400,000 in choline buffer at 0–4° in a 25 × 90 mm centrifuge tube. Ficoll gradients were chosen to circumvent any possible osmotic problems which may have arisen from the use of sucrose gradients. After centrifugation for 1 hr at 100,000 *g* at 0–4° in a swinging bucket rotor, the membrane vesicles at the interface between the 14% and the 16% Ficoll layers were found to represent 90% of the EAA-stimulated $^{22}Na^+$ efflux activity and 63% of the total membrane protein applied to the gradient. The membrane vesicles collected from the interface of the 14% and 16% Ficoll layers of the gradient were diluted to 30 mL with choline buffer at 0–4°, pelleted by centrifugation at 20,000 *g* for 20 min, and resuspended as above in 30 mL of choline buffer at 0–4°. Attempts at more extensive purification by density gradient fractionation resulted in fractionation of the activity with no significant improvement in specific activity.

Whole-particulate membrane vesicle preparation. For collection of the whole-particulate membrane vesicle fraction, the homogenate was diluted to 30 mL with ice-cold Na^+ buffer and centrifuged at 20,000 *g* for 20 min in a fixed angle rotor. The pellet was then resuspended (by homogenization as above) in 30 mL of choline buffer at 0° to yield the final membrane vesicle preparation. There were no detectable differences between the activities of the two different preparations and, therefore, the less purified whole-particular preparation was employed for most of the data reported here.

$^{22}Na^+$ *Efflux assay.* The membrane vesicles, resuspended in choline buffer at 2 mg of membrane protein per mL, were combined with 0.03 vol. of 100 μCi/mL carrier-free [$^{22}Na^+$]:NaCl in water to yield 0.33 μCi/mL of $^{22}Na^+$. The membrane vesicles were incubated at 0° for 150 min to load the membrane vesicles with tracer quantitites of $^{22}Na^+$ (approximately 1 nM Na^+). Following the loading period, 0.5-mL aliquots of the suspension were applied to 2.1-cm Whatman GF/B glass–fiber filters held in a Gooch crucible and subjected to negative pressure from below by means of an adjustable Gast® air pump. The membranes on the filter were washed immediately with three 2-mL aliquots of Na^+ buffer at 25° followed without delay by one 6-mL aliquot of the appropriate concentration of agonist in Na^+ buffer at 25°, followed without delay by three 2-mL aliquots of Na^+ buffer at 25°, all at a flow rate of 2 mL/sec. The filter was then transferred to a 12 × 75 mL polystyrene test tube which was placed in a gamma counter for quantification of the $^{22}Na^+$ retained on the filter. Lowry assay [25] of the filters demonstrated that >90% of the membrane protein applied was retained on the filter through this procedure.

RESULTS

Published descriptions of EAA-stimulated Na^+ fluxes have suggested that this activity was due to glutamate receptor-modulated ionophore activities [26–28]. Therefore, the initial hypothesis of this work was that the activity was not likely to reside in synaptosomes (axon terminals) since most glutamate receptors are localized on dendritic processes [1]. Based on these considerations, it was reasoned that the activity might not copurify with the synaptosomal fraction. Initial experiments were performed on whole-particulate preparations and once a method was established the activity was tracked in density gradients. Ninety percent of the L-glutamic acid-stimulated $^{22}Na^+$ efflux activity from the preparation was collected from the interface of the 14% and 16% (w/v) Ficoll in 130 mM choline chloride buffer layers following density gradient ultracentrifugation. Sixteen percent Ficoll in 130 mM choline chloride is approximately equivalent in buoyant density to 11% Ficoll in 0.32 M sucrose. Membranes isolated from the interface of 7.5% and 13% Ficoll in 0.32 M sucrose (representing a much broader cut than employed here) have been shown to consist of 60–75% synatosomes [29]. It is reasonable to conclude, therefore, that the membrane vesicles which showed the EAA-stimulated $^{22}Na^+$ efflux activity are equivalent to those isolated in conventional synaptosomal preparations. This does not necessarily mean that synaptosomes are the source of the activity. The activity could reside in the remaining 25–40%.

To further address the question of the cellular and subcellular source of the membranes responsible for the EAA-stimulated Na^+ efflux activity, we performed a series of experiments in which veratridine (a Na^+ channel opener) was incorporated into the Na^+ loading buffer. Previous work in our laboratory has shown that 10 μM veratridine increases the total uptake of $^{22}Na^+$ by similar membrane vesicle preparations [30]. Therefore, the effects of veratridine on the loading and subsequent L-glutamic acid-stimulated $^{22}Na^+$ efflux were determined. A typical experimental result with 10 μM veratridine-treated (during $^{22}Na^+$ loading) versus non-veratridine-treated forebrain membranes is presented in Table 1. It was found that veratridine (present during loading of the membranes with $^{22}Na^+$ and to some extent during filtration due to slow washout of veratridine [30]) increased the total amount of $^{22}Na^+$ which could be loaded into the membranes but had no influence on the L-glutamic acid-stimulated efflux component. This demonstrates that the membrane vesicles which exhibit veratridine-dependent Na^+ loading are not the same membrane vesicles which

Table 1. Effects of 10 μM veratridine during loading of rat forebrain membranes with $^{22}Na^+$ on L-glutamate-stimulated $^{22}Na^+$ efflux

	Total cpm remaining on filter*		
	Control	0.1 mM Glutamate	Glutamate-stimulated efflux† (Δcpm)
Membranes loaded without veratridine	4040 ± 50	3400 ± 20	640
Membranes loaded with 1×10^{-5} M veratridine	5570 ± 80	4930 ± 20	640

* Values are means ± SD for three independent determinations.
† Control minus 0.1 mM glutamate.

have EAA-stimulated $^{22}Na^+$ efflux activity. This does not, however, eliminate the possibility that Na^+ channels and EAA-stimulated Na^+ efflux can coexist in the same membrane vesicles.

Incorporation of 1 μM tetrodotoxin (a Na^+ channel blocker) into the wash buffers including the wash containing the EAA agonist, during the Na^+ efflux phase of the experiments, had no detectable effect on any of the treatments including those membranes treated with veratridine. This lack of effect of tetrodotoxin on the efflux of $^{22}Na^+$ stimulated by EAAs further supports the finding that this activity is not mediated by electrogenic Na^+ channels.

Only 37% of the protein from the whole-particulate fraction was separated from the active fraction by density gradient fractionation, and there were no detectable differences in either the EAA-stimulated or background Na^+ efflux activities between the two preparations. Because the preparations were relatively unstable, even at 0–4° (losing activity with a half-time of 1 day), to ensure accuracy of results it was necessary to analyze them on the day they were prepared. The extra 1.5 to 2 hr of preparation time necessary for density gradient fractionation was not offset by any substantive gains in the purity or performance of the preparation and, therefore, the whole-particulate preparation was employed for most of the data reported here.

A number of parameters were tested for this assay system. The method described represents the optimal conditions found based on the criteria of minimizing variance, maximizing the separation of control versus stimulated efflux, and for experimental ease and convenience. Figure 1 represents the influence of time on the loading of the membranes with $^{22}Na^+$ at 0°. One hundred and fifty minutes was chosen as the minimum loading time required to reach equilibrium. Figures 2 and 3 show the influence of time of exposure to the agonist on the stimulated efflux. The data in Fig. 2 indicate that the EAA-stimulated efflux had reached its maximal level by 3 sec. Three seconds represents the minimum time necessary to pass 6 mL of agonist solution through the filter. The decrease in $^{22}Na^+$ remaining on the filters with increasing time is seen to represent control efflux, which is essentially the reverse of loading (Fig. 2).

Since the data in Fig. 2 indicated that the EAA-

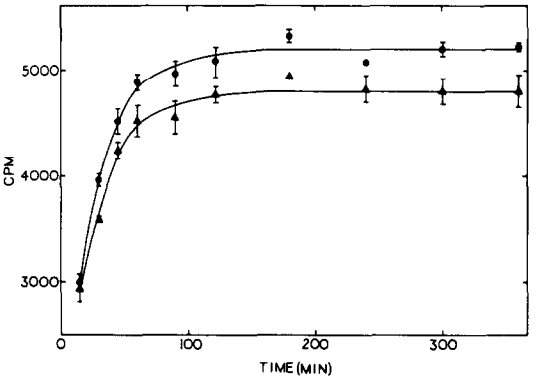

Fig. 1. Time dependence of $^{22}Na^+$ preincubation (sodium loading) on control and L-glutamic acid-stimulated $^{22}Na^+$ efflux. The time of preincubation with $^{22}Na^+$ at 0° was varied with all other aspects of the procedure as described under Materials and Methods. Key: (●) control; and (▲) 50 μM L-glutamic acid. Each point is the mean ± SD of three independent determinations of the amount of $^{22}Na^+$ remaining on the filters.

stimulated efflux had reached its maximum within the 3-sec agonist exposure time of the standard method, it was necessary to modify the method as follows in order to allow measurement of EAA-stimulated efflux at agonist exposure times of less than 3 sec. Membrane vesicles were applied to the filters and washed with three 2-mL aliquots of Na^+ buffer. Then 2 or 4 mL of the agonist solution was applied followed by five, or four, 2-mL washes with Na^+ buffer to yield agonist exposure times of 1 or 2 sec, respectively. It was found that exposures of the membrane vesicles to the agonist solutions for times of less than 3 sec yielded less than the maximal EAA-stimulated efflux (Fig. 3).

To examine the influence of incubation temperature on the EAA-stimulated $^{22}Na^+$ efflux activity, membrane vesicles were prepared as described in Materials and Methods with the exception that all steps were performed at 25° instead of at 0–4°. Employing membrane vesicles which had been prepared and maintained at 25°, it was found that $^{22}Na^+$ retained on the filters following filtration

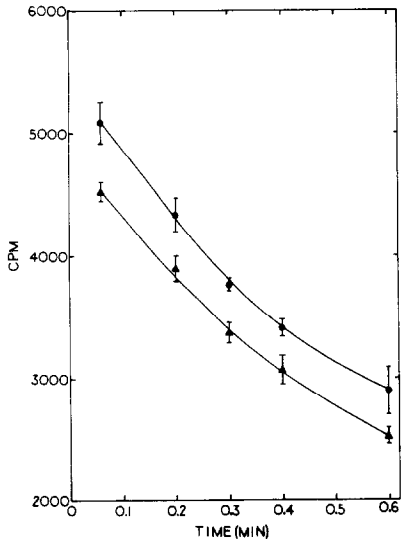

Fig. 2. Influence of increased agonist exposure times on $^{22}Na^+$ efflux. Membranes were applied to the filters and washed with three 2-mL aliquots of Na^+ buffer at 2 mL/sec as described in Materials and Methods. Then 6 mL of the agonist solution or 6 mL of Na^+ buffer for controls was applied, and the filter holder was disconnected from the vacuum system and allowed to flow at gravity pressure for the increased time of exposure to the agonist. This was followed by reapplication of the vacuum and continuation of filtering as described in Materials and Methods. Key: (●) control; and (▲) 50 μM glutamic acid. Each data point is the mean ± SD of three independent determinations of the amount of $^{22}Na^+$ remaining on the filters.

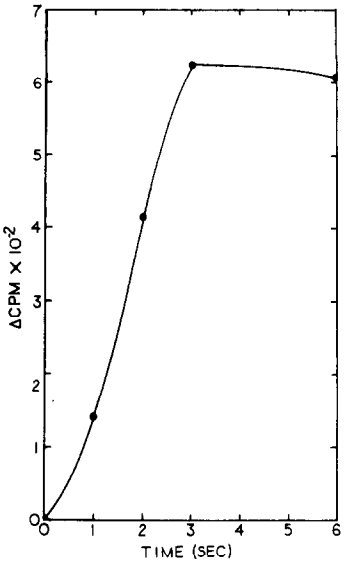

Fig. 3. Influence of decreased agonist exposure times on 50 μM L-glutamate-stimulated $^{22}Na^+$ efflux. Membranes were applied to the filters and washed with three 2-mL aliquots of Na^+ buffer at 2 mL/sec as described in Materials and Methods. Then 2, 4, or 6 mL of 50 μM L-glutamate in Na^+ buffer was applied to the filters followed by five, four, or three 2-mL aliquots of Na^+ buffer. For the 6-sec time of exposure, the filter holder was disconnected from the vacuum system for 3 sec and allowed to flow by gravity as the glutamic acid solution was applied followed by reconnection to the vacuum system and completion of the filtration as for the 3-sec time point.

(as described in Materials and Methods) for the control situation declined with a half-time of approximately 150 min, while EAA-stimulated efflux activity was lost with a half-time of approximately 45 min. The membrane preparation was found to be comparatively stable when maintained at 0–4°, showing >50% of the initial level of glutamic acid-stimulated efflux activity after 24 hr.

The condition of 18 mL total wash volume employed for the standard efflux assay, as described in Materials and Methods, was chosen as the optimum. Fewer washes and lower wash volumes resulted in substantially greater retention of $^{22}Na^+$ on the filters without influencing the EAA-stimulated $^{22}Na^+$ efflux component. Lower wash volumes probably do not effectively remove $^{22}Na^+$ bound to the exterior of the membrane vesicles and the filters. More extensive washing causes gradually decreasing retention of $^{22}Na^+$ by the filters. This probably represents continuing non-stimulated efflux which is most probably the reverse of the loading process due to the increased time necessary for washing.

The influence of buffer composition during filtration and during $^{22}Na^+$ loading was also examined. Replacing some of the Na^+ in the filtration solutions with 5 mM K^+ had no effect on the measurement. Replacing Na^+ with choline in the filtration solutions resulted in approximately a 4-fold higher retention of $^{22}Na^+$ by the filters without measurably affecting the magnitude of the EAA-stimulated component of the efflux (Table 2). This demonstrated that the EAA-stimulated efflux activity was not dependent on high external Na^+ concentrations. Replacing the Na^+ or choline with 130 mM K^+ at any step in the preparation of the membranes or the assay resulted in irretrievable loss of the EAA-stimulated component of the efflux. This suggests that normal membrane polarity is necessary for the EAA-stimulated $^{22}Na^+$ efflux activity.

Replacing some of the choline with Na^+ during the loading of the membrane vesicles with 0.33 μCi/mL $^{22}Na^+$ resulted in decreases in both the L-glutamic acid-stimulated $^{22}Na^+$ efflux (Δcpm) and the total radioactivity retained on the filters (Table 3). Since the membranes employed in this preparation are capable of maintaining a membrane potential [31], these decreases can be seen to be the result of a combination of isotope dilution and membrane potential-dependent effects on Na^+ distribution across the membrane. The total concentration of Na^+ inside the vesicles is expected to not exceed 3–5 mM under any of the conditions employed for $^{22}Na^+$ loading. Thus, with tracer Na^+ only, the $^{22}Na^+$ should be distributed inside and outside the membranes at roughly equal concentrations, while increasing the non-radioactive Na^+ added with the tracer Na^+ should result in progressively smaller

Table 2. Influence of filtration buffer compositions on $^{22}Na^+$ efflux from forebrain membranes

Buffer*	Total cpm remaining on filter†		Stimulated efflux‡ (Δcpm)
	Control	50 μM L-glutamate	
130 mM NaCl	4970 ± 80	4370 ± 70	600
125 mM NaCl + 5 mM KCl	5040 ± 30	4460 ± 50	580
130 mM Choline chloride	19,130 ± 80	18,510 ± 40	620

* In addition to the components listed, all buffers contained 5 mM Tris–HCl, 10 mM glucose, 1 mg/mL BSA at pH 7.4.
† Values are means ± SD for three independent determinations.
‡ Control minus 50 μM L-glutamate.

Table 3. Influence of total sodium concentration during $^{22}Na^+$ loading on the $^{22}Na^+$ efflux measurement

Loading buffer*	Total cpm remaining on filter†		Stimulated efflux‡ (Δcpm)
	Control	50 μM L-glutamate	
130 mM Choline chloride	5800 ± 50	5180 ± 10	620
125 mM Choline chloride + 5 mM NaCl	3210 ± 80	3010 ± 10	200
80 mM Choline chloride + 50 mM NaCl	1770 ± 20	1730 ± 10	40

Efflux was performed with 130 mM sodium buffer throughout as described in Materials and Methods for the standard procedure.
* In addition to the components listed, all buffers contained 0.33 μCi/mL $^{22}Na^+$ as NaCl, 5 mM Tris–HCl, 10 mM glucose, 1 mg/mL BSA, pH 7.4.
† Values are means ± SD for three independent determinations.
‡ Control minus 50 μM L-glutamate.

inside to outside concentration ratios for $^{22}Na^+$ distribution.

Both rat forebrain and hindbrain membranes were prepared and analyzed in the standard $^{22}Na^+$ efflux assay system (described in Materials and Methods). Each preparation showed L-glutamic acid-stimulated $^{22}Na^+$ efflux. The hindbrain preparation showed 65% of the activity of the forebrain preparation based on 1 mg of membrane protein per assay as determined by the method of Lowry *et al.* [25].

As detailed in Table 4, various glutamic acid agonists, antagonists, and related substances were examined for their abilities to stimulate or to inhibit stimulated $^{22}Na^+$ efflux in this preparation. L-Glutamic acid and L-aspartic acid were equal in efficacy and potency in this preparation, typically yielding stimulated efflux values (Δcpm) of approximately 600 with EC_{50} values of 3 μM. KA produced 28% of the maximal response seen with L-glutamic acid or L-aspartic acid with an EC_{50} value of 1.5 μM. QA never showed statistically significant increases in efflux over control experiments nor was $^{22}Na^+$ efflux produced by NMDA in this preparation. Data for L-glutamic acid, KA and QA are given in Fig. 4.

When L-glutamic acid and KA or L-aspartic acid and KA were added to the preparation simultaneously at concentrations of 50 μM for each agonist, the stimulated efflux was the same as for L-glutamic acid or L-aspartic acid alone. L-Glutamic acid and L-aspartic acid added to the preparation simultaneously at concentrations of 50 μM for each agonist yielded stimulated efflux values which were the same as those observed for either agonist alone, demonstrating that neither of these amino acids was capable of augmenting the maximal efflux stimulated by the other. QA (100 μM) or NMDA (100 μM) had no influence on L-glutamic acid-, L-aspartic acid- or KA-stimulation of $^{22}Na^+$ efflux.

Similar to the observations of Chang and Michaelis in their EAA-stimulated $^{22}Na^+$ influx method [32], L-glutamic acid, L-aspartic acid, and KA at concentrations greater than 100 μM were found to show decreased efflux activity compared with lower, optimal concentrations of the same agonist (Fig. 4). KA at 1 mM completely inhibited efflux by 100 μM L-glutamic acid or L-aspartic acid and 1 mM concentrations of these amino acids completely inhibited stimulated efflux due to either of the other two active amino acids (Table 4). The differences in efficacy of KA as an activator at low concentration versus an inhibitor at high concentration suggest that these opposing actions of KA may be due to different effects on the membranes.

Data from radioligand binding studies have demonstrated a requirement for Ca^{2+} in the binding of glutamatergic antagonists of the phosphonate series [33]. APB inhibited up to 40% of the $^{22}Na^+$

Table 4. Influence of various substances on $^{22}Na^+$ efflux from rat forebrain membranes

Substance tested	Concentration for 50% maximum response or maximum concentration tested	Maximum response compared to 100% for 50 μM L-glutamate	Maximum influence on 50 μM L-glutamate-stimulated efflux	Influence on efflux without L-glutamate
L-Aspartic acid	3 μM	100%	No effect	Stimulation
L-Aspartic acid	1 mM	0	100% Inhibition	No effect
Kainic acid	1.5 μM	28%	No effect	Stimulation
Kainic acid	1 mM	0	100% Inhibition	No effect
Quisqualic acid	50 μM	<10%	No effect	Stimulation
APB	1.5 nM	0	40% Inhibition	No effect
APV	100 μM	0	No effect	No effect
L-Glutamic acid diethyl ester	100 μM	0	No effect	No effect
γ-Aminobutyric acid	100 μM	0	No effect	No effect
Glycine	100 μM	0	No effect	No effect
5-Hydroxytyramine	100 μM	0	No effect	No effect
L-Cysteine	100 μM	0	No effect	No effect
L-Cystine	100 μM	0	No effect	No effect
Glycyglycine	100 μM	0	No effect	No effect
Ouabain	1 mM	0	No effect	No effect
Tetrodotoxin	1 μM	0	No effect	No effect

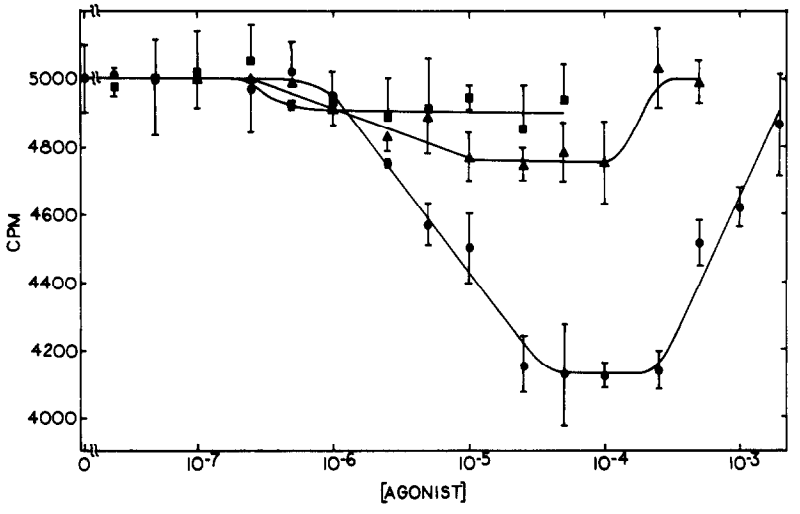

Fig. 4. Concentration–response relationships for L-glutamic acid-, kainic acid- and quisqualic acid-stimulated $^{22}Na^+$ efflux. See Materials and Methods for the details of the experiment. Key: (●) L-glutamic acid; (▲) kainic acid; and (■) quisqualic acid. Each point is the mean ± SD of three independent determinations of the amount of $^{22}Na^+$ remaining on the filters. The units of the abscissa are molar.

efflux stimulated by 50 μM L-glutamic acid or 50 μM L-aspartic acid with an IC_{50} value of approximately 1.5 nM. The data for APB inhibition of L-glutamic acid-stimulated $^{22}Na^+$ efflux are shown in Fig. 5. Calcium was required for the inhibitory action of APB in the efflux assay. APB and 1 mM $CaCl_2$ were incorporated into both the wash buffer and the agonist solutions with all other aspects of the method unchanged. APB was inactive when calcium was omitted from the buffers. Calcium at 1 mM had no influence on the abilities of L-glutamic acid, L-aspartic acid, KA, QA or NMDA to stimulate $^{22}Na^+$ efflux. γ-Aminobutyric acid, glycine, 5-hydroxytyramine, L-glutamic acid diethyl ester, APV, L-cysteine, L-cystine and glycylglycine all at concentrations of 100 μM failed to influence the properties of $^{22}Na^+$ efflux (either control or EAA-stimulated) in this preparation whether calcium was added to the membranes or not (Table 4).

Ouabain (1 mM) incubated with the membrane

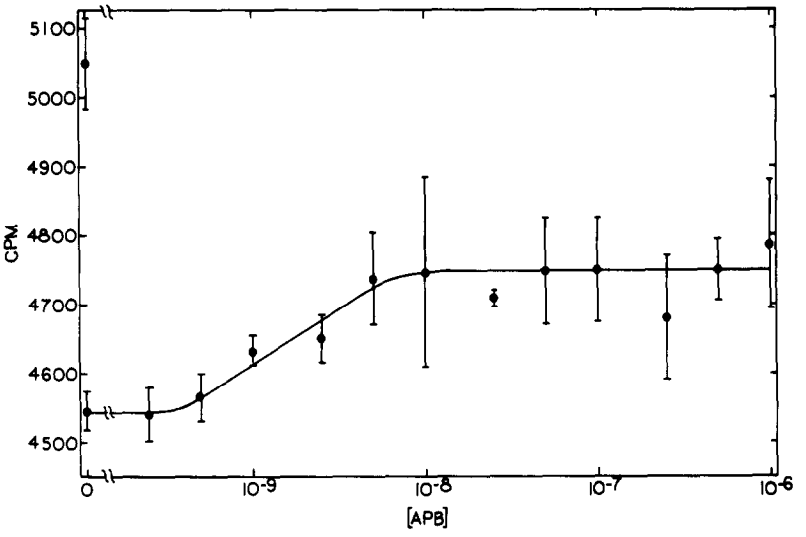

Fig. 5. Inhibition of L-glutamate-stimulated $^{22}Na^+$ efflux by APB. APB and 1 mM $CaCl_2$ were incorporated into the wash buffer and the agonist solution which contained 50 μM L-glutamic acid with all other aspects of the method as described in Materials and Methods. The lone point at the upper left represents the control situation with no L-glutamic acid. Each point is the mean ± SD of three independent determinations of the amount of $^{22}Na^+$ remaining on the filters. The units of the abscissa are molar.

vesicles at 0° for 1 hr after loading with $^{22}Na^+$ and prior to assay under conditions where 1 mM ouabain was incorporated into all filtration buffers did not influence the efflux (Table 4). This demonstrates that Na^+,K^+-dependent ATPase activity is not involved in the EAA-stimulated Na^+ efflux. The possibility remains, however, that Na^+,K^+-dependent ATPase activity may be important for the initial loading of $^{22}Na^+$ into the vesicles.

DISCUSSION

The $^{22}Na^+$ efflux method described here involves superfusion of brain membrane vesicles on glass–fiber filters at a flow rate of 2 mL/sec. The EAA-stimulated $^{22}Na^+$ efflux was found to be complete within 3 sec of exposure of the vesicles to the EAA solution with a half-time of approximately 1.5 sec (Fig. 3). L-Glutamic acid (EC_{50} 3 μM) and L-aspartic acid (EC_{50} 3 μM) were of equal efficacy and were the most efficacious amino acids tested with this procedure. The K_m for glutamic acid transport (3 μM) [18] is the same as the EC_{50} (3 μM) for glutamic acid-stimulated Na^+ efflux reported here. KA (EC_{50} 1.5 μM) produced a maximum of only 28% of the stimulated $^{22}Na^+$ efflux observed for L-glutamic or L-aspartic acid. NMDA and QA at concentrations up to 1 mM were inactive in this preparation either as activators or inhibitors of $^{22}Na^+$ efflux. APB, which has been shown to inhibit chloride-dependent glutamic acid transport processes [17], was found to be a calcium-dependent partial antagonist of L-glutamic acid- or L-aspartic acid-stimulated $^{22}Na^+$ efflux producing a maximum of 40% inhibition of L-glutamic acid- or L-aspartic acid-stimulated efflux with IC_{50} values of 1.5 nM (Fig. 5). APV, which has been shown to be a relatively specific antagonist of NMDA-preferring glutamate receptors while being relatively ineffective at QA-preferring receptors [34], was inactive in this preparation at concentrations up to 1 mM whether calcium was present or not.

Luini *et al.* [26], McIlwain *et al.* [27] and Biziere and Coyle [28] have measured Na^+ fluxes in brain slices in response to L-glutamic acid or other EAAs. These studies employed high concentrations of L-glutamic acid (in the millimolar range), and the ion flux rates observed were either relatively slow or the techniques used were incapable of detecting events occurring on a scale of less than 1 min. Diffusional barriers in the subsurface portions of the tissue slices could account, in part, for reduced ion flux rates and for the elevated EAA concentrations required.

Stallcup *et al.* [35] have measured EAA-stimulated Na^+ influx in a cerebellar nerve cell line which, they concluded, did not have EAA receptors. In their preparation they showed that L-glutamic acid and L-aspartic acid, but not KA or NMDA, were capable of stimulating Na^+ uptake. They also showed that L-glutamic acid exhibited a K_m of 50 μM for this action and they concluded that this activity was due to L-glutamic acid–Na^+ cotransport.

Kimelberg *et al.* [36] have measured EAA-stimulated Na^+ influx with primary astrocyte cultures. They showed that both L-glutamic acid and KA are capable of stimulating Na^+ uptake by the cultured astrocytes, but while L-glutamic acid appeared to stimulate Na^+ uptake, at least in part, by an amino acid cotransport process, KA appeared to be stimulating Na^+ uptake by a different mechanism.

Chang and Michaelis [32] have measured Na^+ influx with synaptosomal and synaptic plasma

membrane vesicle preparations. Their method showed stimulation of $^{22}Na^+$ influx by L-glutamic acid and other EAAs in the same concentration ranges (1 μM for synaptic plasma membrane vesicles) as observed for the $^{22}Na^+$ efflux activity reported here.

In conclusion, this work presents a novel method for detecting and studying EAA-induced movement of Na^+ across CNS membranes *in vitro*. The method measures release of tracer quantities of $^{22}Na^+$, which occurs on a time scale of less than 3 sec, from a membrane vesicle preparation which is sensitive to EAA concentrations in the micromolar range. The results presented allow elimination of electrogenic sodium channels and Na^+,K^+-dependent ATPase activities as direct participants in the EAA-induced sodium efflux activity. However, any one of, and probably a combination of, possible processes including amino acid–sodium cotransport or amino acid–sodium exchange mediated by EAA uptake and/or release processes, or KA receptor-modulated ion channel events, remain as primary mechanisms to explain this activity. Adaptation of this method for use with membranes derived from a variety of cell cultures with defined compositions of EAA-mediated processes for movement of Na^+ across membranes, in combination with further pharmacological manipulations, are seen as the most probable means of fully elucidating the nature of this activity.

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